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14. ABSTRACT
Our objective is to develop a realistic preclinical model of prostate cancer by developing methodology that supports the survival, growth and differentiation of primary cultures of prostate cells in mice. During Year 1, we focused on the method of implantation and the implantation site as the most critical elements in achieving this goal. In Year 2, we took into consideration growing evidence that stem cells are the only cells capable of regenerative growth in vivo, and started developing methodology to isolate stem cells and grow them in primary cultures. We continued these efforts in Year 3, but were not able to accomplish our goal of growing these stem cell primary cultures in vivo because of staffing problems. Staff is now in place and we will resume our in vivo studies under a No Cost Extension in Year 4.

15. SUBJECT TERMS
in vivo model development, primary cultures, epithelial and stromal cells, preclinical model, stem cells

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INTRODUCTION

Currently available models of prostate cancer do not realistically predict activity of experimental therapeutic agents in clinical trials. The objective of our proposed research is to develop a model system that will allow the translation of *in vitro* results to an *in vivo* environment and provide a more realistic preclinical model of prostate cancer than currently exists. Primary cultures, which provide a key *in vitro* model of normal and malignant prostate biology, could fulfill this objective if we can devise a means by which they can be maintained *in vivo* and express appropriate structural and functional differentiation. Our past studies showed that primary cultures transplanted into *nude* mice via standard subcutaneous injection methods rapidly became squamous. We hypothesized that hypoxia is the factor that triggers inappropriate squamous formation that prevents appropriate growth and prostate-specific differentiation of primary cultures *in vivo*. Our experimental plan was to circumvent hypoxia by transplanting cells on a unique gas permeable membrane under the highly vascularized subrenal capsule of the mouse. Our aims were (1) To transplant primary epithelial cell cultures grown on OptiCell™ membranes under the renal capsule of *nude* mice, (2) to transplant primary stromal cell cultures grown on OptiCell™ membranes under the renal capsule of *nude* mice, and (3) to transplant co-cultures of epithelial and stromal cells on OptiCell™ membranes under the renal capsule of nude mice.

In Year One, as described in the first annual progress report (February, 2005), we carried out those Aims and encountered some problems related to the nature of the OptiCell™ membranes. Although primary cultures grew well on the membranes, we found that the membranes were too rigid and provoked a very strong inflammatory and scarring response due to irritation when implanted under the kidney capsule. We concluded that we would have to identify a matrix other than OptiCell™ membranes on which to transplant primary cultures into mice. However, as we developed our experimental strategy for Year Two, we concluded that we could not ignore the developing field of cancer stem cells. Cancer stem cell theory posits that a rare population of functionally distinct cancer cells possesses the extensive self-renewal potential necessary to create a tumor; these are cancer stem cells (CSCs) [1]. Progress in identification of CSCs in other solid tumors including those in the breast and brain has prompted strong belief that prostate cancer is a stem cell disease [2,3]. Cancer stem cell theory further states that only therapies targeting CSCs will effectively render the tumors unable to maintain themselves or grow, thus effecting a cure.

Accordingly, we now believe that our original hypothesis, that hypoxia is the factor limiting growth of primary cultures *in vivo*, is incomplete. While hypoxia may indeed be a critical inhibitory element in the microenvironment, we now believe that primary cultures will never grow and develop appropriate structure and function *in vivo* unless they contain stem cells. Therefore, in Year Two, we devoted our studies to determining whether our primary cultures as historically established do contain a subpopulation of stem cells and, if not, to identifying conditions that permit establishment and growth of stem cells in primary cultures. Our results were described in the Year 2 (February, 2006) progress report. Briefly, we concluded that primary cultures as routinely established did not contain stem cells, but instead consisted of transit amplifying cells (i.e., proliferative basal cells with some progression towards differentiation into secretory cells). We then took some initial steps towards development of

methodologies to isolate and culture stem cells. In that year, we developed techniques to isolate viable single cells that retained cell surface antigens from digested fresh tissues, a prerequisite for using selective techniques to isolate stem cells; showed that a small population of cells expressed CD133, a putative prostate stem cell marker; and demonstrated successful infection with lentivirus of single cells from tissues, another tool that can be used to isolate cells expressing signaling pathways associated with "stemness".

Based on our progress in Year Two, we expected in Year Three to return to our *in vivo* studies but with primary cultures that contained at least a subpopulation of stem cells. However, our progress in Year 3 was not as anticipated due to staffing problems. The research associate working on methodology to culture stem cells was on maternity leave from February to June, then the technician who carries out animal studies left in July. A replacement for the technician has just been found. Therefore, we have asked for and received a No-Cost Extension for an additional year, until January 14, 2008, to complete our studies. Below, we describe our progress to date and our plans to complete the project in the coming year.

BODY

Our first designated task was to transplant primary epithelial cell cultures grown on OptiCell membranes under the renal capsule of nude mice (months 1-12). Our specific goals were to **(a)** prepare epithelial cell cultures on OptiCell membranes in vitro, **(b)** characterize epithelial cells grown on OptiCell membranes in vitro, **(c)** transplant epithelial cells grown on OptiCell membranes in vivo, and **(d)** characterize epithelial cells grown in vivo. We accomplished all components of this aim, as described in the first annual progress report (February, 2005). We concluded that the OptiCell membranes were not suitable as an implantation platform because they caused an undesirable inflammatory response. The membranes, composed of a proprietary plastic, are rather rigid and presumably cause physical trauma to the kidney, hence the inflammatory reaction and scar formation.

Our second aim was to transplant primary stromal cell cultures grown on OptiCell™ membranes under the renal capsule of nude mice (months 13-20). Our specific goals were to **(a)** prepare stromal cell cultures on OptiCell membranes in vitro, **(b)** characterize stromal cells grown on OptiCell membranes in vitro, **(c)** transplant stromal cells grown on OptiCell membranes in vivo, and **(d)** characterize stromal cells grown in vivo. Although our original intent had been to not initiate studies with stromal cells until Year Two, we decided to carry out experiments in conjunction with the epithelial cells in Year One, and these results were also described in the first annual progress report. Histologic analysis of the implanted membranes revealed the same phenomenon as noted in the experiments with epithelial cells. Even the OptiCell membrane itself with no cells caused inflammation and extensive scar formation, as was also seen with the membranes carrying cells. This validated our conclusion from the previous studies that the membranes acted as an irritant in the kidney and this property precludes their utility as a platform for implantation of cell cultures.

Our third aim was to transplant co-cultures of epithelial and stromal cells on OptiCell™ membranes under the renal capsule of nude mice (months 21-36). Our specific goals were to **(a)** co-culture epithelial and stromal cells on OptiCell membranes in vitro, **(b)** characterize co-

cultures of epithelial and stromal cells grown on OptiCell membranes in vitro, **(c)** transplant co-cultures of epithelial and stromal cells on OptiCell membranes in vivo, and **(d)** characterize co-cultures of epithelial and stromal cells in vivo. Although we had not planned to initiate co-culture experiments until Year 3, we decided to start some of these experiments since we were already working with epithelial and stromal cells in Aims 1 and 2. Carrying out this Aim required in vitro studies to first identify optimal co-culture conditions, which were described in the first annual progress report. We did not attempt to implant any co-cultures on OptiCell membranes into nude mice given the problem with inflammation that we encountered in Aims 1 and 2.

At the end of Year One, we concluded that OptiCell membranes would not provide a suitable platform for implantation of primary cultures of prostatic cells under the renal capsule of nude mice. We expected to devote Year Two to identifying and testing other substrates for implantation. However, as alluded to in the Introduction, we postponed those studies. Instead, we devoted Year Two to testing methodology for the primary culture of prostate cancer stem cells. Our results were described in the Year Two Progress Report (February, 2006) and are briefly recanted below:

(1) Search for presence of stem cells in primary cultures established according to standard methodology. We concluded that our primary cultures as routinely established did not have stem cells because they lacked the stem cell properties of infinite self-regeneration, were incapable of anchorage-independent growth, and did not contain any CD133-positive cells (a putative stem cell marker). We then initiated a series of experiments aimed at altering our traditional primary culture methodology in order to establish primary cultures containing stem cells.

(2) Isolation of single cells from human prostate cancer tissues. Stem cells are often identified by the expression of specific cell surface antigens and sorted by flow cytometry. Therefore, in order to culture stem cells, it will be necessary to culture single cells. This is challenging for cells originating from glandular epithelium such as that of the prostate, since such cells prefer to be maintained as acini and attach as an aggregate. Our standard protocol for establishment of primary cultures involves digestion of tissues to acini, but not to single cells, because we have found that single cells do not attach or grow well.

Subsequently, we proceeded to optimize a protocol to generate a good single cell suspension from prostate cancer tissue. The optimal protocol that we developed involves a 2-4 hr digestion of minced tissue with medium containing high concentrations of collagenase I and hyaluronidase to release prostatic acini, and a short (5-10 min) digestion with 0.2% trypsin/0.2% EDTA to release single cells from the acini. We typically obtain an average of 1-2 x 10^5 prostatic cells/0.1g tissue. The trypsinization doesn't destroy cell surface antigens as shown by immunolabeling with antibody against epithelial cell-specific antigen (ESA).

(3) Evidence of CD133-positive cells from freshly digested tissues. CD133 is a promising prostate stem cell marker currently under investigation. We examined the expression of CD133 in single cells freshly dissociated from prostate cancer tissue using the protocol described above and flow cytometry. Single cells generated from a fresh cancer specimen after surgery were

stained with Phycoerythrin (PE)-conjugated CD133 antibodies. A distinct rare population (2.2%) of $CD133^+$ cells existed in the tumor specimen. Compared to a normal tissue specimen, there was a more than 5-fold enrichment of $CD133^+$ cells in the cancer specimen, consistent with the hypothesis that CSCs arise from the dysregulation of self-renewal of normal stem cells and therefore CSCs are in a greater number than their normal counterparts. These results demonstrated that CD133 is expressed by a rare population of cells in prostate cancer, characteristic of stem cell markers. In vitro culture of the cells generated from fresh tissue in the standard serum-free medium that our lab uses for primary culture of prostatic epithelial cells resulted in a loss of $CD133^+$ cells. Similarly, $CD133^+$ cells were not detected by flow cytometry in several primary prostate epithelial cell cultures established previously in our lab. In Year Three, described below, we attempted to culture CD133-positive cells.

(3) Expression of GFP in prostate cells by lentivirus infection. Wnt signaling through the canonical β-catenin pathway has been shown to regulate stem cell renewal in several tissues [4]. Activation of TCF4-driven gene expression has been shown to be a direct downstream target event of the activation of Wnt signaling pathway, which is important for the maintenance of stem cells. Therefore, it is conceivable that stem cells in the prostate may also require TCF4-mediated gene expression to maintain their stemness. To select cells that have activated TCF4, we used GFP as a reporter and delivered TCF4-GFP, a construct in which GFP is linked to a promoter with three TCF4 binding sites, into cells by lentivirus infection. A control construct was used in which GFP is downstream of the same promoter except that the TCF4 binding sites are inactivated by mutations. Lentivirus carrying these constructs were produced in 293T cells, and concentrated by ultracentrifugation at a titer of 3.66×10^7 TU/ml. In prostate epithelial cells digested from fresh tissues, lentivirus infection led to expression of GFP under the control of the phosphoglycerate kinase (PGK) promoter at a level comparable to that in HCT116 cells (positive control), demonstrating the feasibility of delivering TCF4-GFP into these cells using lentivirus.

We infected single cells generated from a prostate cancer specimen with lentivirus carrying either wild type TCF4-GFP or mutated TCF4-GFP, and analyzed the GFP expression using flow cytometry. Approximately 12% of the cells infected with wild type TCF4-GFP showed considerably higher level of GFP compared to cells infected with mutated TCF4-GFP, demonstrating the existence of a small population of cancer cells with activated TCF4-mediated gene expression. We also examined CD133 expression in these infected cells, and found that the differences in CD133 expression between isotype control and antibody-stained cells were minimal, consistent with our previous observation that in vitro culture under standard conditions resulted in a loss of CD133 expression in CSCs or a loss of CSCs all together.

(4) Primary culture of single cells. After demonstrating that we could create a population of viable single cells that retained cell surface antigens from digested fresh prostate cancers, our next goal was to identify conditions that would permit attachment and growth of these single cells *in vitro*. We first tested our standard primary culture conditions, which include collagen-coated dishes and the serum-free medium "Complete PFMR-4A" [5]. While these conditions are optimal for the attachment and growth of acini, single cells did not attach or grow in these conditions. We proceeded to test a number of other media and substrates and found success by using a feeder layer of stromal cells (mouse 3T3 cells) and a newly commercially available

defined medium from CellNTec (CnT-12). Complete PFMR-4A also supported attachment and growth of single cells in conjunction with a feeder layer; while growth in Complete PFMR-4A was perhaps not quite as good as in CnT-12, Complete PFMR-4A does offer the advantage that we know the constituents, unlike the commercial medium. While we do not yet know if any of these colonies were derived from stem cells, the ability to generate colonies from single cells represents the first required step towards culturing stem cells.

(5) $\gamma c^-/RAG2^-$ mice as an in vivo model for identification of CSCs. To obtain definitive evidence of the existence of CSCs, an *in vivo* functional analysis must be established. It is essential to determine whether a population of cells can initiate tumors *in vivo* in order to distinguish tumorigenic vs. non-tumorigenic cells. Various research groups have attempted to establish xenograft models of fresh, histologically intact human prostate cancer tissues in immunodeficient mice. The low engraftment rate experienced by these groups can be attributed to two main factors, the host environment and the grafting site. Several xenotransplantation models were developed based on severe combined immunodeficient (SCID) mice and their derivative, the non-obese diabetic (NOD)/SCID mouse model. The utility of the existing SCID mouse models is limited due to several disadvantages, including some "leakiness" that results in the appearance of mature B/T lymphocytes and immunoglobulins, residual natural killer (NK) cell activity, and a high rate of spontaneously developing thymomas that limit their lifespan. A new SCID mouse model has been developed by crossing mice lacking the common cytokine receptor γ chain for interleukin (IL)-2, IL-4, IL-7, IL-9, and IL-15 with mice lacking the recombinase activating gene-2 (RAG-2). The offspring has a stable phenotype characterized by the absence of all T and B cells and NK function. This novel immunodeficient mouse proved to be useful for studying xenotransplantation of human plasmacytoid dendritic cell precursors and human peripheral blood lymphocytes for the development of a severe acute graft-vs.-host disease model [6]. It also has been widely used by Dr. Weissman's lab and other stem cell investigators at Stanford with great success. The group has been astounded at the growth of enriched glioblastoma stem cells in this host. Glioblastomas, like prostate cancer, have traditionally proven very difficult to grow as xenografts in mice. Enriched stem-like cells from head and neck cancers, as well as from ovarian cancers, have also been growing well in the $\gamma c^-/RAG2^-$ mice.

We made our first attempt to implant prostate cancer cells under the renal capsule of two $\gamma c^-/RAG2^-$ mice. Cells were suspended in an equal volume of Matrigel and kept on ice until they were injected. The surgery was performed as described by Wang et al. [7] during a 20 min period for each mouse during which sterile practices were followed throughout. The mice were then kept in a designated area in the Stanford Research Animal Facility. Both mice survived the procedure, and there were no signs of infection.

Progress in Year Three

(1) Optimization of cell sorting by flow cytometry. Since putative stem cells are often selected by expression of particular cell surface antigens (e.g., CD133) or signaling pathways (e.g. Wnt), it is imperative to be able to sort viable, rare cells from single cell populations obtained from freshly digested tissues. In Year Two, we found that we could isolate viable single cells from digested tissues that retained cell surface antigens or were infectable by lentiviruses. We found evidence of CD133-positive cells by FACS analysis

and evidence of GFP-lentivirus expression driven by Wnt signaling. We also found that single cells could be cultured using a 3T3 feeder layer and CnT-12 medium. Next, we wanted to see if we could sort single cells by FACS that would retain viability and grow in culture. This proved to be a challenge. When we either sorted cells by CD133 expression or GFP expression after lentivirus infection, they did not grow in culture. Additional experiments showed that cells were not viable after sorting. We have learned from colleagues that stem/epithelial cells from solid tumors, as opposed to hematopoietic cells, are difficult to sort because they are very fragile. Over time, we have worked with the flow cytometer facility to modify nozzle size and flow force in an effort to cause less cell damage; optimization is still in progress. Other problems with flow cytometry have included contamination and difficulty in scheduling (because we never know exactly what time surgery will be completed). In an effort to avoid these problems, we also tested cell separation with antibody-conjugated magnetic beads, but we were dissatisfied with the inadequate separation of different cell populations.

(2) Cell isolation based on biological properties. Given our problems with cell isolation by FACS, we focused on other putative properties of stem cells. One of these is the ability to exclude the dye Hoescht 33342. This is the so-called "side population" of cells that express the ABCG2 transporter that actively excludes drugs and dyes [8]. Since Hoescht 33342 is toxic, cells that can't exclude the dye (non-stem cells) die, whereas the dye-excluding stem cells live. First, we used our standard primary cultures as examples of non-stem cells to determine their sensitivity to Hoescht 33342. Cells were treated with 0.5-5.0 μg/ml of the dye for 1 hour, then tested for their growth potential. The ID50 was ~1.5 μg/ml. Next, we isolated single cells from a digested cancer tissue and treated half of the cells with Hoescht 33342. The other half was untreated as a control. The cells were then inoculated onto 3T3 feeder layers in medium CnT-12. The dished inoculated with the treated cells (putatively containing dye-resistant stem cells) yielded a greater number of colonies than the untreated population (suggesting selection of stem cells with greater clonogenic potetial?). The dye-resistant colonies also had a different morphology from the untreated colonies. We have repeated this experiment several times now with cells from different cancer tissues, with similar results. Serial passage of the dye-resistant cells and the control populations are now in progress to determine if the dye-resistant cells have the stem cell-associated property of infinite self-regeneration.

Plans for the no-cost extension in Year 4

Now that we have personnel in place again, we will return to our in vivo studies. We will implant putative prostate cancer stem cells under the renal capsule of $\gamma c^-/RAG2^-$ mice. Initially, we will start with total populations of single cells digested from fresh tissues. We can use these to determine the best conditions to promote cell viability and growth (i.e., co-injection with Matrigel or collagen or stromal cells, etc.). The $\gamma c^-/RAG2^-$ mice have not previously been used for prostate cells, so we anticipate some novel results. Then, we will use our selected putative stem cell populations. Currently, Hoescht 33342-resistant cells look most promising, but as we optimize our flow sorting methodologies, we may have additional populations to test as well. We will do short-term assays initially, and recover the injected cells after a week or so to examine viability, structure and function. If these findings are

positive, then we will carry out long-term experiments to assess growth and transplantability. Our goal is to be able to maintain stem cells back and forth between culture and in vivo, which has not yet been accomplished by anyone for prostate cancer stem cells isolated directly from fresh tissues.

KEY RESEARCH ACCOMPLISHMENTS

- Initiated experiments to optimize flow cytometry sorting of putative stem cells followed by in vitro cultivation
- Isolated and cultured putative stem cells that are resistant to Hoescht 33342

REPORTABLE OUTCOMES

None.

CONCLUSIONS

Science is dynamic, and we have had to modify our original hypothesis as new information has developed. Our original premise was that primary cultures of prostate cancer cells would be capable of tumor formation *in vivo* if provided with the appropriate environment. We hypothesized that the appropriate environment would be under the renal capsule, where hypoxia would not be present and would not provoke growth-limiting, inappropriate squamous differentiation. We know believe that hypothesis to be too simplistic, because it did not take into account recently accumulating knowledge regarding the existence and characteristics of cancer stem cells. Accordingly, our hypothesis must now posit that growth *in vivo* will rely not only on the nature of the host environment but also on the nature of the primary cultures themselves. Last year, we developed the tools and techniques required for primary culture of prostate cancer stem cells. Next year, we will return to our goal of identifying optimal techniques for tumor formation of these primary cultures *in vivo*. At the conclusion of our studies, we expect to provide a model system of *in vitro* and *in vivo* propagation of the cells most relevant to prostate cancer therapy, i.e., prostate cancer stem cells.

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APPENDICES

None.